ALKALINE LIPASE FROM VIBRIO METSCHNIKOVII RH530 N-4-8 AND NUCLEOTIDE SEQUENCE ENCODING THE SAME

BACKGROUND OF THE INVENTION

This application claims priority from Korean Patent Application No. 2002-35410, filed on June 24, 2002, in the Korean Intellectual Property Office, the disclosure of which is incorporated herein in its entirety by reference.

1. Field of the Invention

The present invention relates to an alkaline lipase isolated from *Vibrio metschnikovii* RH530 N-4-8 and a gene encoding the same. The present invention also relates to a recombinant vector containing the gene, a transformed host cell transformed with the recombinant vector and a method of producing an alkaline lipase using the transformed host cell.

2. Description of the Related Art

An alkaline lipase hydrolyses triacylglycerol into glycerol and fatty acid at alkaline pH. Various microorganisms producing an alkaline lipase have been reported. Specifically, representative examples of such microorganisms include *Pseudomonas*, and *Bacillus*. These enzymes have been applied to industrial fields of detergents that necessitate hydrolysis of lipids under alkaline conditions.

Currently, lipases for commercially available detergents biochemically exhibit an optimal activity at weak alkaline pH, that is, at pH 8~9, and are relatively rapidly inactivated in the presence of an anionic surfactant, e.g., LAS.

Thus, there is demand for lipases exhibiting an optimal activity at a higher pH level, e.g., at pH 10~11, a high ratio of residual enzyme activity and high compatibility with surfactants.

In order to overcome problems with prior art, inventors of the present invention found out that *Vibrio metschnikovii* RH530 N-4-8 (on deposit at the Korean Collection for Type Culture (KCTC) with

KFCC-11030 on February 23, 1998), which is a strain producing protease for a detergent, as disclosed in Korean Patent laid-open Nos. 10-1996-0007772 and 10-1999-0084319, also produced a lipase. They intensively studied biochemical properties of the lipase, a gene encoding the lipase and its resistance to a surfactant and completed the present invention.

SUMMARY OF THE INVENTION

The present invention provides a lipase exhibiting an optimal activity at a high pH level, that is, at pH 10~11, and having a high residual enzyme activity and high compatibility with a surfactant.

According to an aspect of the present invention, there is provided gene encoding the lipase.

According to another aspect of the present invention, there is provided recombinant vector containing the gene encoding the lipase.

According to another aspect of the present invention, there is provided transformed host cell transformed by the recombinant vector.

According to another aspect of the present invention, there is provided a method of producing the lipase by cultivation of the host cell.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other features and advantages of the present invention will become more apparent by describing in detail exemplary embodiments thereof with reference to the attached drawings in which:

FIG. 1 shows a recombinant vector pHL1 containing 3.2 kb DNA insert (*val*L) having an alkaline lipase gene according to the present invention;

FIG. 2 shows an agarose gel electrophoresis of the recombinant vector pHL1 having an alkaline lipase gene according to the present invention, in which M denotes a size marker, lane 1 has a supercoiled type pUC19, lane 2 has a pUC19 digested with *Hind*III, lane 3 has a recombinant vector pHL1 digested with *Hind*III, the band of 2.7 kb corresponding to a vector pUC19 and the band of 3.2 kb corresponding

to a DNA insert containing the alkaline lipase gene according to the present invention, and lane 4 has a supercoiled type recombinant vector pHL1;

FIG. 3A shows an agarose gel electrophoresis of a DNA fragment containing the alkaline lipase gene according to the present invention, and FIG. 3B shows a photograph of Southern blotting, in which M denotes a size marker marked by DIG, lane 1 has *Vibrio metschnikovii* chromosomal DNA, lane 2 has *Vibrio metschnikovii* chromosomal DNA digested with *Hind*III, lane 3 has *Vibrio metschnikovii* chromosomal DNA digested with *Ava*I and *Eco*RI, lane 4 has pUC19 digested with *Hind*III, lane 5 has a supercoiled type recombinant vector pHL1, lane 6 has a recombinant vector pHL1 digested with *Hind*III, and lane 7 has recombinant vector pHL1/*Ava*I and *Eco*RI (probe);

FIGS. 4A and 4B show a base sequence of a DNA insert containing the alkaline lipase gene from *Vibrio metschnikovii* RH530 N-4-8 according to the present invention, a regulatory element and an amino acid sequence derived therefrom;

FIG. 5 shows a restriction enzyme map from which a minimum length and a gene position for expression of the alkaline lipase according to the present invention are identified in the DNA insert of the recombinant vector pHL1;

FIG. 6 shows the comparison result of an amino acid sequence deduced from the alkaline lipase gene according to the present invention with *Pseudomonas glumae*, and B*urkholderia cepacia*;

FIG. 7A shows a restriction enzyme map of a region prior to the promoter of the alkaline lipase gene according to the present invention, and FIG. 7B shows a change in activity when the region prior to the promoter is removed using the restriction enzyme;

FIG. 8A shows a change in activity of the alkaline lipase according to the present invention, and FIG. 8B shows the measuring result of residual activity of the alkaline lipase according to the present invention depending on temperature;

FIG. 9A shows a change in activity of the alkaline lipase according to the present invention depending on pH, and FIG. 9B shows the measuring result of residual activity of the alkaline lipase according to the present invention depending on pH; and

FIG. 10 shows the effect of surfactant or detergent on the activity and stability of the alkaline lipase according to the present invention, for which enzyme solutions mixed with sodium-olefinsulfonate (AOS) (FIG.10A), sodium alkylbenzen-sulfonate (LAS)(FIG. 10B) and sodium dodecyl sulfate (SDS)(FIG. 10C) are spotted on a 0.5% tricaprylin medium.

DETAILED DESCRIPTION OF THE INVENTION

An alkaline lipase from *Vibrio metschnikovii* RH530 N-4-8 according to the present invention has an amino acid sequence of SEQ ID NO: 5.

Also, a polynucleotide according to the present invention encodes an amino acid sequence of SEQ ID NO: 5. The polynucleotide includes polynucleotide containing a nucleotide sequence of SEQ ID NO: 4, polynucleotide containing a nucleotide sequence of SEQ ID NO: 2 and a nucleotide sequence of SEQ ID NO: 4, for example. Also, the polynucleotide may have a nucleotide sequence of SEQ ID NO: 1.

A recombinant vector according to the present invention includes a polynucleotide encoding an amino acid sequence of SEQ ID NO: 5, preferably a polynucleotide having a nucleotide sequence of SEQ ID NO: 4. Preferably, the recombinant vector is pHL1, pHLB29 or pHAAH38.

A transformed host cell according to the present invention is transformed by the recombinant vector including a polynucleotide encoding an amino acid sequence of SEQ ID NO: 5, preferably a polynucleotide having a nucleotide sequence of SEQ ID NO: 4. The recombinant vector is preferably pHL1, pHLB29 or pHAAH38. Preferably, the transformed host cell is *E.coli* transformed by the recombinant vector. The transformed *E.coli* is preferably HB101 harboring pHL1.

A method of producing the alkaline lipase from *Vibrio metschnikovii* RH530 N-4-8 according to the present invention includes culturing the transformed host cell.

Also, a detergent according to the present invention includes an alkaline lipase from *Vibrio metschnikovii* RH530 N-4-8 having an amino acid sequence of SEQ ID NO: 5. The detergent is preferably in a liquid or particulate form.

The present invention will now be described in more detail with reference to various embodiments. These embodiments are provided for illustration only and the invention is not limited to the specific embodiments.

EXAMPLES

The examples of the present invention are based on the finding that *Vibrio metschnikovii* RH530 N-4-8, which is known as a strain producing protease for a detergent, as disclosed in Korean Patent laid-open Nos. 10-1996-0007772 and 10-1999-0084319, produces a lipase. Biochemical properties of the lipase, a gene encoding the lipase and its resistance to a surfactant have been intensively studied.

In order to isolate a gene encoding an alkaline lipase, *Vibrio metschnikovii* RH530 N-4-8 was cultured to collect a cell. The cell was lysed with lysozyme treatment. The resultant product was treated with phenol and chloroform to remove protein and was subjected to centrifugation to remove precipitate, giving a supernatant. *Vibrio* chromosomal DNA was obtained from the supernatant. The obtained chromosomal DNA was cut with a restriction enzyme and inserted into a cloning vector pUC19, producing recombinant vectors including pHL1, pHLB29 and so on, which was transformed into *E. coli*. Screening for right clones was performed with LB media containing 0.5~1% tributyrin or tricaprylin as a lipase substrate, 0.1% polyoxyethylene (7EO) as an emulsifier. 1.8% agarose were added to produce a medium shown in Table 1 and a strain forming a clear halo around a colony grown in the medium was selected. The thus selected recombinant *E. coli* was

referred to as HB101(pHL1).

The activity of a lipase was measured using a crude enzyme solution extracted from the recombinant *E. coli* HB101(pHL1) at a weak alkaline pH, confirming expression of an alkaline lipase.

The recombinant vector was cut with a restriction enzyme and a base sequence of heterogenous DNA fragment inserted into the recombinant vector.

Example 1: Cloning of alkaline lipase gene

Vibrio metschnikovii RH530 N-4-8 was cultured at 30℃ using the culture medium shown in Table 1 to collect a cell and treated with lysozyme to lyse the cell. The resultant product was treated with phenol and chloroform to remove protein, and a precipitate was removed by centrifugation, giving a supernatant. A *Vibrio* chromosomal DNA was obtained from the supernatant. The obtained chromosomal DNA was cut with a restriction enzyme *Hind*III to be recombined with cloning vector pUC19, followed by transforming *E.coli* HB101, thereby cloning a DNA fragment containing a 3.2 kb alkaline lipase gene. The resulting recombinant vector was referred to as a vector pHL1 (FIG. 1). After treatment with the restriction enzyme *Hin*dIII, an electrophoresis with 1% agarose gel was performed. The agarose gel electrophoresis showed that the alkaline lipase gene was cloned (FIG. 2).

Table 1. LSC Medium

Composition	Content (g/L)
Trypton	10
Yeast extract	5
Sodium chloride	10
1M Sodium carbonate buffer, pH 10.5	100 (mℓ/L)

Example 2 : Southern blotting of pHL1

In order to confirm that a DNA fragment containing an alkaline lipase gene derived from *Vibrio metschnikovii*, which is contained in a recombinant vector pHL1 shown in FIG. 1, is identical with the gene from *Vibrio metschnikovii*, Southern blotting was performed.

A 0.8 kb DNA fragment labeled with DIG(DIG DNA Labelling Kit, Roche Diagnostics), which was obtained by cutting pHL1 using restriction enzymes *Ava* I and *EcoR* I, was used as a probe, and blotted with a chromosomal DNA extracted from the original strain, that is, *Vibrio metschnikovii* RH530 N-4-8, resulting in a colored band at 3.2 kb, as shown in FIGS. 3A and 3B.

It was confirmed that a gene contained in the recombinant vector pHL1 was derived from RH530 N-4-8 (FIGS. 3A, 3B and 4)

Example 3 : Subcloning for verifying position of lipase gene

In order to verify an exact position of a gene in the DNA inserted into a recombinant vector, 3.2 kb DNA was treated with an exonuclease *Bal*31 to subclone the same in a minimum length required for expression of a lipase.

Production of the lipase was confirmed by formation of a clear halo, and the result of subcloning showed that 2.6 kb DNA fragment was necessary for lipase activity. The recombinant vector containing such a gene having a minimum length was referred to as pHLB29.

2.6 kb DNA fragment was subcloned in a direction opposite to that of a *Sma* I site of pUC19, and referred to as pHAAH38.

Although the 2.6 kb DNA fragment was subcloned in a reverse direction relative to a *lac* promoter, pHAAH38 produces a clear halo at a tricaprylin culture medium, confirming that an alkaline lipase promoter exists in the 2.6 kb DNA fragment and the promoter is used when it is transcribed from *E.coli* (FIG. 5).

Example 4: Production of recombinant vector for base sequence analysis

A DNA insert of the recombinant vector pHL1 produced above was cut into smaller sizes using various restriction enzymes, and recombined again with pUC19 for transformation of *E. coli*. A base sequence analysis of the DNA insert in the recombinant vector showed that the base sequence of SEQ ID NO:1 was identified, and two genes ORF1 and ORF2 respectively consisting of 797bp (SEQ ID NO:2) and

554bp (SEQ ID NO:4) existed under single promoter. Enzymes expressed from the genes were referred to as Val L1 and Val L2, and genes encoding the same were referred to as *val*L1 and *val*L2, respectively. The *val*L1 and *val*L2 genes have base sequences of SEQ ID NOS: 2 and 4, and the polypeptide encoded by the genes have amino acid sequences of SEQ ID NOS: 3 and 5. It was also found that they had base sequences corresponding to sites –35 and –10 and a Shine-Dalgarno sequence (SD sequence) (FIG. 4), which are commonly found in a prokaryotic gene. Sequences of these sites and other lipase sequences were compared in view of homology, and the comparison result showed that the second gene was homologous with *Pseudomonas glumae* and *Burkholderia cepacia* lipase genes by 17.5% and 18.3%, respectively. Also, as shown in FIG. 6, the gene had a region corresponding to an active site of a lipase, that is, G-X1-S-X2-G. Thus, it is considered that the gene is a lipase gene, and the first gene is lipase chaperon or an auxiliary gene for extracellular secretion.

Example 5: Measurement of activity and stability of alkaline lipase

The activity of enzyme was measured using a synthetic substrate p-nitrophenyl palmitate (pNPP), rather than using emulsified natural oil. First, 20 $\mu\ell$ of a crude enzyme solution obtained by culturing mother stain, *Vibrio metschnikovii* RH530 or a recombinant strain containing lipase gene, was added to a 880 $\mu\ell$ of a buffer solution containing 50 mM tris-HCl (pH 6.8) and 0.5% Arabic gum. Then, 100$\mu\ell$ of a 100 mM p-NPP solution was added to the resultant solution and reacted at 37℃ for 10 minutes. After 10 minutes, 0.5 ml of 3M HCl was added to stop the reaction, followed by centrifuging, adding 3 ml of 2M NaOH to 1 ml of a supernatant. Then, absorbance was measured at 420 nm.

In an alternative method, p-nitrophenyl butyrate (p-NPB) was used as a substrate. First, p-MPB was dissolved in dimethylsulfoxide to prepare a 10mM substrate solution. 30$\mu\ell$ of the substrate solution was mixed with a buffer solution containing 50mM tris-HCl and 0.1%

triton-X-100 (pH8.2), 30$\mu\ell$ of a crude lipase solution was added thereto, giving 3 ml of a final product. The final product was also reacted at 37℃ for 10 minutes, and then 3 ml acetone was added to stop the reaction. Then, the absorbance was measured at 405 nm.

Quantitative analysis of protein was based on bovine serum albumin (BSA) using a Lowry's method.

Example 6: Study of effect of a region prior to the promoter on enzyme expression

In order to investigate effects of a region prior to the promoter on expression of *Vibrio metschnikovii* alkaline lipase, the region prior to the promoter was removed using restriction enzymes. For measuring enzyme activity, p-nitrophenyl butyrate (p-NPB) was used as a substrate and *BamH*I and *Afl*III were used as restriction enzymes. As a result, it was confirmed that removal of 500 bp of the region prior to the promoter resulted in a reduction in enzyme titer by approximately 40%.

Therefore, it could be inferred that expression of an enzyme was greatly influenced by the region prior to the promoter (FIGS. 7A and 7B).

Example 7: Biochemical properties of lipase extracted from recombinant strain

In order to investigate effects of temperature, pH, surfactant or detergent on the lipase activity, the following experiments were carried out on crude enzyme solutions prepared from recombinant strains containing lipase genes.

(1) Effect of temperature on activity and stability

In order to investigate effects of temperature on the activity and stability of an enzyme, *E. coli* HB101 harboring pHL1 containing a lipase gene was cultured in a culture medium shown in Table 1 for 18 hours to collect a cell. Then, the collected cells were washed twice using saline, and pulverized using a sonicator or French press, followed by centrifuging at 15,000 rpm for 30 minutes, giving a supernatant. The obtained supernatant was used as a crude enzyme solution. The crude enzyme solution was mixed with p-NPB and reacted over various

temperature ranges from 10℃ to 80℃ for 2 hours. Then, the activity and stability of the lipase were measured by the above-described titer measuring technique. The measurement result showed that the lipase exhibited highest activity at 50~60℃. Also, the result of residual activity testing showed that the stability increased up to 40℃ and then rapidly decreased from 60℃ (FIGS. 8A and 8B).

(2) Effect of of pH on the activity and stability

In order to investigate effects of a pH level on the activity and stability of an enzyme, the ratio of residual enzyme activity of the crude enzyme solution extracted in the above-described manner was examined under the same reaction conditions at optimal pH levels in various buffers, including 50 mM sodium phosphate (pH 6～7), as indicated by ◆, 50 mM Tris-HCl (pH 7～9): as indicated by ■, 50mM sodium carbonate (pH 9～11), as indicated by ▲, and 50 mM sodium phosphate-NaOH (pH 11～12), as indicated by *. The result showed that the optimal pH was 10～11. To examine the residual enzyme activity ratio relative to pH level, each crude enzyme solution was allowed to stand at 20℃ for 12 hours, and then the residual activity was measured. The result showed that the enzyme was very stable at pH in the range of 8～10(FIG. 9B).

(3) Effect of surfactant on activity and stability

In order to measure resistance against a surfactant, which is a main component of a detergent, sodium-alphaolefinsulfonate (AOS), sodium alkylbenzen-sulfonate (LAS), sodium dodecyl sulfate (SDS) were mixed with the crude enzyme solution, followed by spotting the mixture on a 0.5% tricaprylin culture medium.

The result showed that the *Vibrio* alkaline lipase had resistance against 0.07% LAS and 0.1% AOS.

Also, the *Vibrio* alkaline lipase was active in 0.1% SDS, confirming that the lipase can be suitably used as an additive for a laundry detergent (FIG. 11).

Biochemical properties of general lipases for detergents currently

commercially available in the market will now be described. That is, the lipases exhibit an optimal activity at pH 8~9, that is, a weak alkaline level, and are relatively rapidly inactivated in the presence of an anionic surfactant such as LAS. On the other hand, the lipases according to the present invention exhibit an optimal activity at pH 10~11 and had very high ratio of residual enzyme activity and high compatibility with a surfactant. Thus, the lipase according to the present invention is considered to be better than the conventional lipase in view of performance and can be suitably used as an enzyme for a laundry detergent.

Deposit of recombinant vectors produced in the Examples of the present invention

The recombinant vectors pHL1 and pHLB29 produced in the present invention were on deposit at the Korean Culture Collection Center (KCCM) with KCCM-10384 and KCCM-10385 on June 4, 2002.

Industrial Applicability

According to the present invention, the alkaline lipase has an optimal activity at high pH level, that is, at pH 10~11, a very high ratio of residual enzyme activity and high compatibility with a surfactant, so that it can be suitably used as an enzyme for a laundry detergent.

The gene according to the present invention encodes an alkaline lipase having low homology with other conventional alkaline lipases, an optimal activity at high pH level, that is, at pH 10~11, a very high ratio of residual enzyme activity and high compatibility with a surfactant, the alkaline lipase being suitably used as an enzyme for a laundry detergent.

While the present invention has been particularly shown and described with reference to exemplary embodiments thereof, it will be understood by those of ordinary skill in the art that various changes in form and details may be made therein without departing from the spirit and scope of the present invention as defined by the following claims.